REMARKS

Claim 1-9 have been canceled.

Claims 10-14 are pending.

Claims 15-19 have been added. Claim 15 is an independent claim, i.e., it is not dependent on claim 10. It is requested that claim 15 be examined on its own merit.

Claim 10 has been amended by adding limitations requiring the proliferation inducing peptide be a viral transformation peptide, and that the fusion protein induce proliferation of terminally differentiated cells.

It is respectfully brought to Examiner's attention that functional claim limitations are proper and cannot be ignored.

A functional limitation is an attempt to define something by what it does, rather than by what it is (e.g., as evidenced by its specific structure or specific ingredients). There is nothing inherently wrong with defining some part of an invention in functional terms. Functional language does not, in and of itself, render a claim improper. In re Swinehart, 439 F.2d 210, 169 USPQ 226 (CCPA 1971). A functional limitation must be evaluated and considered, just like any other limitation of the claim, for what it fairly conveys to a person of ordinary skill in the pertinent art in the context in which it is used. MPEP § 2173.05(g).

No amended or new claims add new matter.

Examiner is respectfully reminded that the Applicants' earliest priority date is July 15, 1999.

Rejection Under 35 USC § 112

The final office action of March 3, 2004 asserts that the claims are not enabled by the specification. Applicants respectfully disagree.

Examiner states that the claims do not provide any guidance or examples to indicate the disclosed product as a tissue regenerating agent in a patient.

A.

For the purpose of expediting the prosecution of the claims, claim 10 has been amended to recite an agent of inducing terminally differentiated cells to divide. However, it is noted that Examiner has improperly read limitations into the claims that were not recited. There is not any claim language that refers to a product or indicates a patient. By reading these limitations into claims the Examiner is rendering judgments beyond the scope of the Patent Office's jurisdiction.

Specifically, whether or not the agent is a marketable product that can be used in a patient is the province of the FDA, and not the PTO. However, the currently amended claim 10 renders this issue moot.

B.

Examiner also states that the claims "read *in vitro* and *in vivo* use." The Examiner is again reading limitations into the claims. However, in this case the Examiner is assessing patentability of the claimed agent as if it were a method claim. Clearly, this is not proper claim interpretation.

The claim is to an agent with the biological property of inducing terminally differentiated cells to enter the cell cycle. Compounds, isolated nucleic acids and proteins are routinely patented without being limited to *in vivo* and/or *in vitro* activities. This is the case even when the compounds or nucleic acids, etc are disclosed as being for pharmaceutical use.

Attached are pages from patents that issued last week, i.e., August 24, 2004. These patents have no *in vivo* data, yet the main claims are not limited to *in vitro* applications. This would likely not be the case if the instant claims were method claims.

In sum, the rejection for lack of an enabling disclosure should be withdrawn. The claims are clearly enabled in the manner exemplified by the attached patent excerpts.

C.

Examiner cites the Durer article showing a predominantly nuclear accumulation of the fusion protein to support his argument that the fusion proteins "may not accumulate in the correct subcellular compartment."

This conclusion is completely unsupported by any evidence of record, and thus, constitutes mere speculation that the fusion protein may not be useful.

> A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. See, e.g., In re Marzocchi, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. Wertheim, 541 F.2d at 263, 191 USPQ at 97.

MPEP § 2164.04.

Examiner has not carried the burden sufficient to reject the claims for lack of enablement. Examiner's speculation that the fusion proteins would not be properly sorted intracellularly, and especially, that the allegedly missorted protein would be ineffective/inoperable, is not sufficient to maintain the rejection.

The Derer article also states that the terminally differentiated myotubes actually reentered the cell cycle. See legend to figure 1, and text of column 2, page 133. Therefore, assuming for argument's sake that Examiner's speculation was well-reasoned, it is empirically shown to be incorrect and cannot be sufficient to maintain the rejection. See the attached additional Derer article; Derer II.

In sum, the amended claims, as well as the new claims, should be allowed as they are clearly enabled and supported by the specification.

D.

Claim 10 has been narrowed to encompass a small subgroup of proteins that induce proliferation of terminally differentiated cells.

New independent claim 15 specifically limits the claim to T-ag as the component (b) of the fusion protein.

It is suggested that the amended claims are adequately described in the specification and the knowledge in the art. Accordingly, withdrawal of the rejection is requested.

Rejections Under § 102

In the previous response, Applicants pointed out that Braetge was not anticipatory prior art because it did not provide a disclosure that enabled the claims.

In a telephone conversation with Examiner he asserted that a cited patent need not provide an enabling disclosure because once it issues it is presumed enabled. Apparently, Examiner believes this presumption applies to that everything a reference discloses. Therefore, he maintained the rejections under § 102 by Braetge based on a Full Faith and Credit theory, which is not even referred to in the MPEP.

It has been long standing practice that any prior art must be enabling. The MPEP unambiguously states the following:

> "In determining that quantum of prior art disclosure ***which is necessary*** to declare an applicant's invention 'not novel' or 'anticipated' within section 102, ***the stated test is whether a reference contains an 'enabling disclosure'...*** ." In re Hoeksema, 399 F.2d 269, 158 USPQ 596 (CCPA 1968). A reference contains an "enabling disclosure" if the public was in possession of the claimed invention before the date of invention. "Such possession is effected if one of ordinary skill in the art could have combined the publication"s description of the invention with his [or her] own knowledge to make the claimed invention." In re Donohue, 766 F.2d 531, 226 USPQ 619 (Fed. Cir. 1985).

MPEP § 2121.01 (emphasis added).

The undersigned independently established the veracity and relevance of this rule in a telephone discussion with Supervisor Gary Kunz on or about June 10, 2004. In this conversation Supervisor Kunz explicitly confirmed that a prior art reference must be

enabling. In this context, Braetge's disclosures cannot reasonably be considered anticipatory prior art.

The specific issues raised in either of Braetge's references include, *inter alia*, the following:

- Braetge does not describe *any* actual constructs having T antigen.
- Braetge does not describe any method of joining a translocation peptide and T antigen.
- Braetge does not disclose the relative positioning of the proteins in the fusion; which would be upstream/downstream? Also, see Braetge '735, col. 5, lines 60-65 where he discloses that it is important whether a protein is attached to VP22's amino or carboxyl terminus.
- Braetge provides no evidence that any of his fusion proteins even possess the required property of being able to translocate itself into a cell from the medium (see below).
- Braetge does not disclose even one fusion protein that induces the cell's immortalization, let alone induce proliferation of a differentiated cell.

Braetge has only demonstrated the synthesis of his constructs, when expressed from plasmids transfected into Cos-1 cells. There is no evidence in Braetge demonstrating that *any* of his constructs actually immortalize cells. In fact, just the opposite. See example in col. 16 lines 32-38 disclosing that osteosarcoma cells become apoptotic; i.e., the cells die rather than become immortalized.

Further, Braetge does not demonstrate that any of his fusion proteins can translocate from the outside, e.g., the culture medium, to the inside of cells when contacted with the fusion protein. Braetge only shows that transfected Cos-1 cells express a fusion protein. This does not disclose the protein's translocatability into the cell, because each cell that stains with antibodies was transfected with the expression plasmid. In other words, Braetge's cells are making the protein intracellulary.

Therefore, there is no guidance whatsoever in Braetge that would provide persons of ordinary skill in the art sufficient guidance to provide a functioning fusion

protein as claimed by Applicants. As shown in the Applicant's articles, e.g., Drerer II, attached, Applicants employ the fusion protein *per se*, added exogenously to the myotube media to demonstrate its effectiveness.

Respectfully, Braetge cannot reasonably be found to satisfy the rule of *Hoeksma*: that a reference contains an "enabling disclosure" if the public was in possession of the claimed invention before the date of invention. Braetge cannot reasonably be viewed as placing into the public's possession, functional fusion proteins exemplified by VP22-T antigen, that induce terminally differentiated cells to divide.

Accordingly, Braetge cannot be anticipatory prior art solely based on a mere reference to a protein comprising VP22 and SV40 T antigen.

It is respectfully requested that the anticipation rejection over the Braetge references be withdrawn.

CONCLUSION

It is respectfully requested that the rejections under §§ 102(e) and 112, 1st paragraph be withdrawn in view of the foregoing amendments and remarks. It is believed that allowance is proper in view of the narrowed scope of the amended and new claims. Further, it is believed that Braetge's patents have been shown not to provide disclosures that enable the making or using of the claimed subject matter.

Allowance is respectfully solicited.

CONDITIONAL PETITION FOR EXTENSION OF TIME

If any extension of time for this response is required, Applicants request that this be considered a petition therefore. Please charge the required fee to Deposit Account No. 14-1263.

ADDITIONAL FEES

Please charge any further insufficiency of fees, or credit any excess to Deposit Account No. 14-1263.

Respectfully Submitted,

Norris, McLaughlin & Marcus
220 East 42 nd Street
New York, NY 10017
Telephone (212) 808-0700
Facsimile (212) 808-0844

Theodore Gottlieb,PhD
Reg. No. 42, 597

Certificate of Mailing

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Hon. Commissioner of Patents P.O. Box 1450 Alexandria, VA 22313-1450

on August 30, 2004.
(Date)

Agata Glinska
Typed or printed name of person signing this certificate

Signature Agata Glinska

The FASEB Journal express article 10.1096/fj.01-0500fje. Published online November 29, 2001.

A novel approach to induce cell cycle reentry in terminally differentiated muscle cells

Wolfgang Derer, Hariharan P. Easwaran, Heinrich Leonhardt and M. Cristina Cardoso

Max Delbrück Center for Molecular Medicine, 13125 Berlin, Germany

Corresponding author: M. Cristina Cardoso, Franz Volhard Clinic, Wiltbergstr. 50, D-13125 Berlin, Germany. E-mail: cardoso@fvk-berlin.de.

ABSTRACT

During terminal differentiation, skeletal muscle cells permanently retract from the cell cycle. We and others have shown previously that this cell cycle withdrawal is an actively maintained state that can be reversed by transient expression of the SV40 large T antigen. In an attempt to avoid the hazards of gene transfer and the difficulties of regulating transgene expression, we have now used this cellular system as a model to test whether direct protein delivery could constitute a feasible alternative or complementing strategy to gene therapy-based approaches. Taking advantage of the recently described intercellular trafficking properties of the herpes simplex virus I VP22 protein, we have constructed a chimeric VP22-SV40 large T antigen fusion protein and shown that it can spread into terminally differentiated myotubes where it accumulates in the nucleus. This fusion protein retains the ability to override the cell cycle arrest as shown for SV40 large T antigen alone. Our results clearly show that the transduced fusion protein remains capable of inducing S-phase and mitosis in these otherwise terminally differentiated cells and opens now the way to exploit this novel strategy for tissue regeneration.

Key words: protein therapy • SV40 large T antigen • VP22

Terminal differentiation of neuronal and muscle cells is the main obstacle preventing tissue regeneration in mammals, including man. Skeletal myogenesis is used widely as a model system to study cell cycle regulation during terminal differentiation and to develop new strategies to reverse this process. Skeletal myoblasts proliferate in culture and can be induced to differentiate under mitogen deprivation conditions. This differentiation process can be monitored unequivocally as myoblasts fuse and form characteristic multinucleated myotubes, which express muscle-specific proteins and are withdrawn permanently from the cell cycle (1).

We and others have shown previously that the cell cycle arrest of terminally differentiated muscle cells is an actively maintained process that can be reversed on the transient expression of the simian virus 40 (SV40) large T antigen oncoprotein (2–5). For these studies the C2C12 mouse skeletal muscle cell line (6) was transfected stably with a thermolabile SV40 large T antigen mutant under the control of the mouse metallothionein I gene promoter (3). Induction of the SV40 large T antigen in this C2SVTts cell line by addition of Zn^{2+} and shifting to the permissive temperature of 33°C results in cell cycle reentry and, to a small extent, apoptosis (4).

One mechanism by which the SV40 large T antigen induces cell cycle reentry in terminally differentiated muscle cells is via its interaction with the retinoblastoma gene product (5). In fact, the retinoblastoma protein (pRb) has been shown to be essential for maintaining cell cycle arrest in terminally differentiated skeletal muscle cells because Rb-deficient myoblasts can fuse into myotubes expressing myogenic markers but, on growth factor stimulation, reenter the cell cycle (7, 8). It has also been shown that pRb is down-regulated in mitotic nuclei after retrodifferentiation (9). It is clear that inactivation of pRb function is required for cell cycle reentry. However, phosphorylation of pRb protein, which abrogates its E2F suppressive activity, does not suffice to allow cell cycle reentry in myotubes (10).

These experiments demonstrated that terminal differentiation can be overcome by transfection with an oncogene, which, however, leads to a genetic modification of target cells. Although the expression of the oncogene can be controlled with inducible promoters, it might still get inserted downstream of an endogenous promoter. Although the frequency of such an unfortunate insertion might be low, it is still too high a risk to be acceptable for human therapy, as a single transformed cell could be sufficient to start a lethal tumor. These problems could be avoided by applying the gene product meaning the protein rather than the gene itself.

Recently the herpes simplex virus I (HSV-1) tegument protein VP22 was described to have the remarkable property of intercellular trafficking. Once expressed in a transfected cell, the protein is exported and transferred via an actin cytoskeleton-dependent mechanism to the surrounding recipient cells where it accumulates in the nuclei (11). Even when fused to heterologous proteins, VP22 retains its ability of intercellular spreading, thereby acting as a vehicle to cargo fused proteins into target cells. Accordingly, a VP22-p53 fusion protein was used successfully to induce apoptosis in a p53 deficient osteosarcoma cell line (12). To date, VP22 fusion proteins have been shown to spread in several established lines of proliferating cells (13), and we have shown that VP22 can cargo fused GFP into terminally differentiated skeletal muscle cells (14).

In this work, we investigated whether it is in principle possible to overcome terminal differentiation by protein transduction. We tested whether the SV40 large T antigen can be delivered directly via VP22 to C2C12 myotubes and whether this direct oncoprotein transfer into myotubes is sufficient to stimulate cell cycle reentry.

MATERIALS AND METHODS

Construction of VP22-TAg expression plasmid

We amplified the SV40 large T antigen by polymerase chain reaction (PCR) from pCMVTAgOri vector (kind gift from P. Löser, Max Delbrück Center for Molecular Medicine (Berlin)) with primers flanking the open reading frame and the PCR product was cloned into the pVP22myc/hisTopo vector (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. Transcription is under the control of the cytomegalovirus promoter (CMV) and the fusion protein (VP22-TAg) contains at its C-terminal end a myc and his epitope tags.

Cell culture, transfection, and replication labeling

COS-7 and C2C12 cells were grown as described previously (14). C2C12 myoblasts were seeded onto gelatin-coated coverslips and myogenic differentiation was induced as described (14) by changing the growth media (Dulbecco's modified Eagle's medium [DMEM] containing 20% fetal calf serum [FCS]) to differentiation media (DMEM supplemented with 5% horse serum) and incubating at 37°C for 3–4 days.

For immunoblot analysis, COS-7 cells were transfected with 1 μg VP22-large T antigen plasmid DNA or mock transfected by using the diethylaminoethyl-dextran pretreatment method as described previously (15). Forty-eight hours after transfection, the cell monolayers were washed in phosphate buffered saline (PBS) and scraped, and whole cell extracts were prepared and analyzed.

For coculture experiments, COS-7 cells were transfected with the VP22-large T antigen construct using GenePorter Transfection Reagent (Gene Therapy Systems, San Diego, CA). We diluted 7 μg plasmid DNA in 1 ml serum-free DMEM and mixed it with 1 ml DMEM containing 35 μl GenePorter reagent. After aspirating the media from a p60 culture dish with COS-7 cells, this solution was added to the cells. After 5 h, we added 2 ml DMEM containing 20% FCS. After another 24 h, the COS-7 cells were trypsinized and transferred to C2C12 myotube cultures. The mixed cultures were kept in DMEM containing 20% FCS. After 24, 48, and 72 hours, the cocultures were fixed either for 5 min in ice-cold methanol or for 15 min in 3.7% formaldehyde and analyzed by immunofluorescence staining. DNA synthesis was monitored by adding 5-bromo-2'-deoxyuridine (BrdU) to the culture media to a final concentration of 100 μM 24 h before fixing the cells.

Antibodies

The following primary antibodies were used: anti-myc tag mouse monoclonal antibody (clone 9E10); anti-his tag mouse monoclonal antibody (Dianova, Hamburg, Germany); anti-SV40 large T antigen mouse monoclonal antibody (PAB 101); anti-PCNA rabbit polyclonal antibody (FL 261, Santa Cruz Biotechnology, Santa Cruz, CA); and anti-BrdU rat monoclonal antibody (clone BU1/75, Harlan Sera Lab, Sussex, U.K.). For immunoblot analysis, we used horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Buckinghamshire, U.K.).

For immunofluorescence analyses, we used the following secondary antibodies: Cy5 or Texas red-conjugated anti-mouse IgG; biotinylated anti-rat IgG; FITC-conjugated anti-rabbit IgG (all from Jackson ImmunoResearch, West Grove, PA).

Immunoblot analysis

Transfected COS-7 cells were extracted for 30 min on ice in RIPA buffer as described (16). In brief, we analyzed cell extracts and cell pellets by immunoblot using anti-myc tag mouse monoclonal antibody to detect the VP22-Tag fusion protein.

Immunofluorescence

Formaldehyde-fixed cultures were permeabilized further with 0.5% Triton X-100/1% sodium dodecylsulfate in PBS. Cultures were then washed and incubated for 1 h with diluted primary antibodies, followed by detection with fluorochrome-conjugated secondary antibodies as described earlier (16). To detect BrdU incorporation during DNA replication, the rat anti-BrdU antibody was incubated together with DNAse I at 37°C to allow antibody access to the halogenated base. After being washed with PBS, cultures were incubated with biotinylated anti-rat IgG, followed by FITC or Texas red-labeled streptavidin (Amersham). Total DNA was counterstained with Hoechst 33258 (Sigma, St. Louis, MO), and samples were mounted in mowiol.

The stained cultures were examined with an Axioplan 2 or Axiovert 100TV microscopes (Zeiss, Göttingen, Germany) equipped with phase contrast and epifluorescence optics, by using a 63× Planapochromat oil immersion objective NA 1.4 and bandpass FITC, Texas Red, Cy5, and Hoechst filter sets. Images were collected with a cooled CCD camera (Sensicam, PCO Computer Optics GmbH, Kelheim, Germany) using the Axiovision software (Zeiss) and assembled with Adobe (San Jose, CA) Photoshop and Illustrator software.

RESULTS

Transgenic expression of SV40 large T antigen has been shown to induce reversal of terminal differentiation and cell cycle reentry in skeletal muscle cells (2–5).

In order to test whether direct protein delivery could also induce retrodifferentiation, we constructed a translational fusion in which the SV40 large T antigen containing a C-terminal his/myc tag was fused in frame at the C-terminus of HSV-1 VP22 under the control of the cytomegalovirus promoter (Fig. 1A). Western blot analysis of protein extracts of VP22-large T antigen transfected COS-7 cells indicated a band at the expected size of 120 kDa (Fig. 1B). Immunofluorescence analysis of VP22-large T antigen transfected COS-7 cells with an antibody recognizing the his tag revealed the typical localization pattern described for the GFP-VP22 fusion (11). Transfected cells showed cytoplasmic localization, whereas surrounding nontransfected cells accumulated the VP22-large T antigen fusion protein in the nuclei (data not shown).

To analyze the trafficking ability of the VP22-large T antigen chimeric protein into skeletal muscle cells, we performed coculture experiments of transfected COS-7 cells with terminally differentiated C2C12 myotubes (Fig. 2A). The VP22-large T antigen transfected COS-7 cells were trypsinized and transferred to the myotube culture. Cocultures with mock transfected COS-7 cells, which express the large T antigen without VP22, were performed as a negative control. After 24, 48, and 72 h, the mixed cultures were fixed with methanol or formaldehyde and immunostained for his tag or SV40 large T antigen to detect the presence of transducing VP22-TAg protein in myotubes. Figure 2B depicts a myotube stained for the his tag, which accumulated the VP22-large T antigen fusion protein in its nuclei. No his signals were detected in the negative controls consisting of cocultures of mock-transfected COS-7 cells with myotubes (data not shown). To control for the eventuality that the wild-type SV40 T antigen stably

expressed by COS cells could be transferred to myotubes, we stained the cocultures of myotubes with VP22-TAg or mock-transfected COS-7 cells with an antibody recognizing T antigen. Figure 2C shows micrographs of these stained cocultures. Myotubes that were cocultured with VP22-large T antigen expressing COS-7 cells (upper row) accumulated the chimeric fusion protein in the nuclei (Fig. 2C) as seen before with the anti-his tag antibody (Fig. 2B). In the mock-transfected COS cell control (lower row), the myotube nuclei were completely free of SV40 large T antigen, whereas the endogenous COS cell large T antigen was easily detectable in all COS cell nuclei. These data clearly indicate that large T antigen can be transferred into the myotube nuclei only by using VP22 as a vehicle.

To address the question of whether the delivered large T antigen is still active and able to induce cell cycle reentry even in fusion with VP22, we assayed myotubes cocultured for 24, 48, or 72 h with VP22-large T antigen expressing COS-7 cells for incorporation of BrdU into replicating DNA and induction of proliferating cell nuclear antigen (PCNA), which is necessary for DNA replication and not expressed in differentiated myotubes (2). Using antibodies raised against SV40 large T antigen (mouse monoclonal antibody), BrdU (rat monoclonal antibody), and PCNA (rabbit polyclonal antibody), we performed indirect immunofluorescence stainings of the cocultures, which had been incubated with BrdU for 24 h before formaldehyde fixation. Microscopical analysis revealed that the VP22-TAg was indeed able to induce S-phase reentry as seen by the positive BrdU signals and induction of the replication factor PCNA. Figure 3 shows an example of a myotube that accumulated VP22-large T antigen in its nuclei. This direct delivery of the chimeric protein resulted in S-phase reentry as indicated by induction of PCNA and incorporation of BrdU into the replicating DNA. In contrast, no BrdU or PCNA positive nuclei were detected in the control myotubes cocultured with mock-transfected COS-7 cells (data not shown). Furthermore, Figure 4 depicts a metaphase plate in a myotube nucleus, showing that VP22-large T antigen can induce mitosis in terminally differentiated muscle cells.

DISCUSSION

In this study, we aimed to explore a novel approach to reverse terminal differentiation. We took advantage of the ability of HSV-1 VP22 to transduce fused proteins into cells and tested whether direct protein delivery of SV40 large T antigen via fusion to VP22 could induce cell cycle reentry in terminally differentiated muscle cells. We show here that VP22 can cargo SV40 large T antigen into terminally differentiated skeletal muscle cells (Fig. 2), which as a consequence reenter the mitotic cell cycle (Figs. 3 and 4).

In addition to viral oncogenes, another attractive candidate is the msx1 transcriptional repressor, which was shown recently to induce C2C12 myotube dedifferentiation (17). Future experiments will show whether other combinations of regulatory factors, including msx1, can be delivered with this approach and can elicit a regenerative response. This protein delivery strategy could be combined easily with the application of other compounds; for example, myoseverin, a newly reported small microtubule-binding purine shown to induce myotube cytokinesis (18).

This approach also offers new possibilities for investigating the effects of other factors in terminally differentiated skeletal muscle cells. Because these cells resist most gene-transfer

methods [our unpublished results and (19)] the direct protein delivery provides a novel alternative to apply regulatory factors in a time- and dose-controlled manner to living cells.

Finally, VP22-derived particles, designated Vectosomes, have shown been shown recently to cargo proteins as well as nucleic acids into cells where they remain stable until they are released by light stimulation (20). This light-induced release of cargo could be used for a temporally and spatially controlled delivery of therapeutic factors.

ACKNOWLEDGMENTS

W. D. was supported by a research training program for clinicians (Klinische Ausbildungsprogramm, KAP) from the Max Delbrück Center for Molecular Medicine (Berlin). This work was supported by the Deutsche Forschungsgemeinschaft and by the Max Delbrück Center for Molecular Medicine.

REFERENCES

1. Nadal-Ginard, B. (1978) Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* **15**, 855–864

2. Cardoso, M. C., Leonhardt, H., and Nadal-Ginard, B. (1993) Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. *Cell* **74**, 979–992

3. Endo, T. and Nadal-Ginard, B. (1989) SV40 large T-antigen induces reentry of terminally differentiated myotubes into the cell cycle. In *The Cellular and Molecular Biology of Muscle Development* (Kedes, L. H., and Stockdale, F. E., eds.) pp. 95–104, Alan R. Liss, Inc.: New York

4. Endo, T. and Nadal-Ginard, B. (1998) Reversal of myogenic terminal differentiation by SV40 large T antigen results in mitosis and apoptosis. *J. Cell Sc.* **111**, 1081–1093

5. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell* **72**, 309–324

6. Yaffe, D. and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* (*London*) **270**, 725–727

7. Novitch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996) Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J. Cell Biol.* **135**, 441–456

8. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994) Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. *Science* **264**, 1467–1471

9. Endo, T. and Goto, S. (1992) Retinoblastoma gene product Rb accumulates during myogenic differentiation and is deinduced by the expression of SV40 large T antigen. *J. Biochem.* **112**, 427–430

10. Mal, A., Chattopadhyay, D., Ghosh, M. K., Poon, R. Y., Hunter, T., and Harter, M. L. (2000) p21 and retinoblastoma protein control the absence of DNA replication in terminally differentiated muscle cells. *J. Cell Biol.* **149**, 281–292

11. Elliott, G. and O'Hare, P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**, 223–233

12. Phelan, A., Elliott, G., and O'Hare, P. (1998) Intercellular delivery of functional p53 by the herpesvirus protein VP22. *Nat. Biotechnol.* **16**, 440–443

13. Wybranietz, W. A., Prinz, F., Spiegel, M., Schenk, A., Bitzer, M., Gregor, M., and Lauer, U. M. (1999) Quantification of VP22-GFP spread by direct fluorescence in 15 commonly used cell lines. *J. Gene Med.* **1**, 265–274

14. Derer, W., Easwaran, H. P., Knopf, C. W., Leonhardt, H., and Cardoso, M. C. (1999) Direct protein transfer to terminally differentiated muscle cells. *J. Mol. Med.* 77, 609–613

15. Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* 71, 865–873

16. Cardoso, M. C., Joseph, C., Rahn, H. P., Reusch, R., Nadal-Ginard, B., and Leonhardt, H. (1997) Mapping and use of a sequence that targets DNA ligase I to sites of DNA replication in vivo. *J. Cell Biol.* **139**, 579–587

17. Odelberg, S. J., Kollhoff, A., and Keating, M. T. (2000) Dedifferentiation of mammalian myotubes induced by msx1. *Cell* **103**, 1099–1109

18. Rosania, G. R., Chang, Y. T., Perez, O., Sutherlin, D., Dong, H., Lockhart, D. J., and Schultz, P. G. (2000) Myoseverin, a microtubule-binding molecule with novel cellular effects. *Nat. Biotechnol.* **18**, 304–308

19. Nalbantoglu, J., Pari, G., Karpati, G., and Holland, P. C. (1999) Expression of the primary coxsackie and adenovirus receptor is downregulated during skeletal muscle maturation and limits the efficacy of adenovirus-mediated gene delivery to muscle cells. *Hum. Gene Ther.* **10**, 1009–1019

20. Normand, N., van Leeuwen, H., and O'Hare, P. (2001) Particle formation by a conserved domain of the herpes simplex virus protein VP22 facilitating protein and nucleic acid delivery. *J. Biol. Chem.* **276**, 15042–15050

21. Elliott, G. and O'Hare, P. (1998) Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. *J. Virol.* **72**, 6448–6455

Received August 13, 2001; accepted September 28, 2001.

Fig. 1

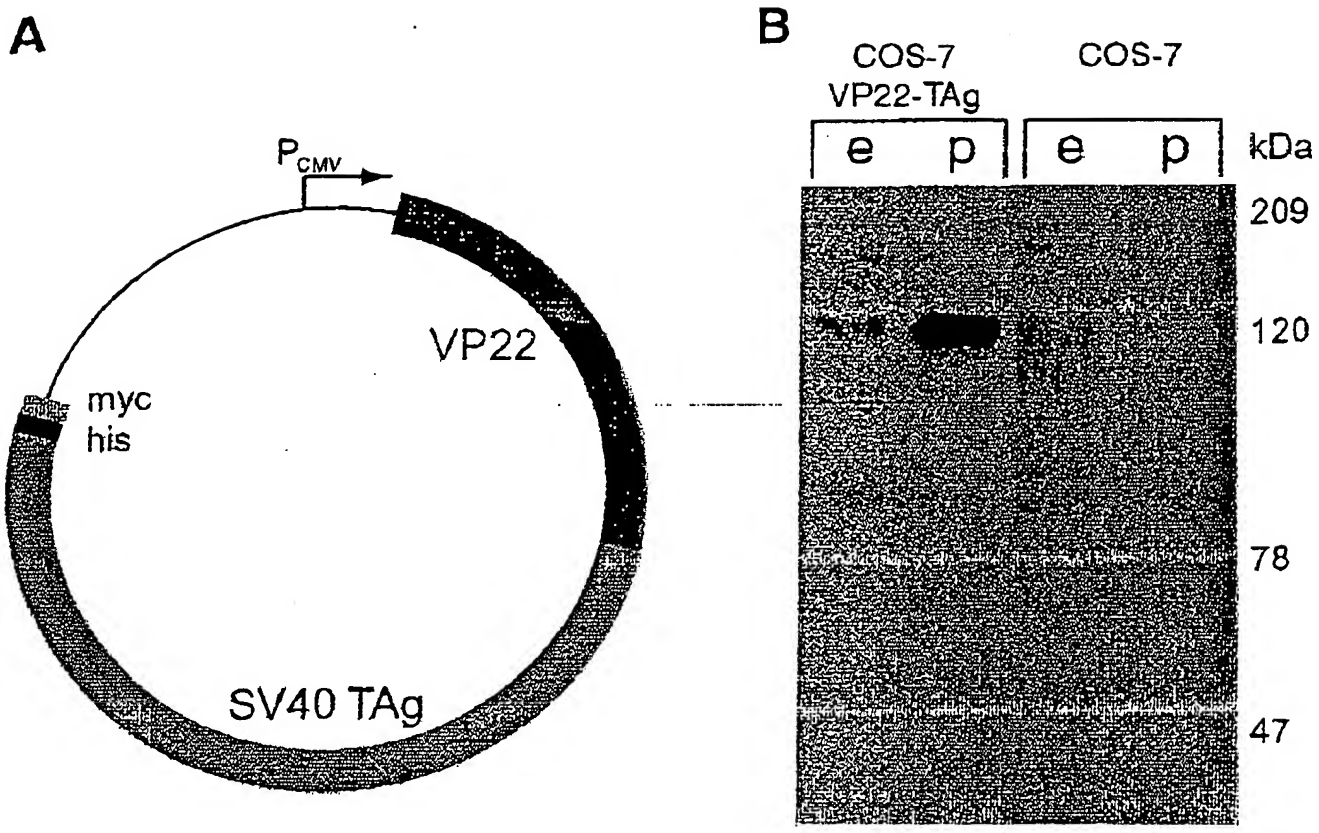

Figure 1. Construction and characterization of the VP22-large T antigen expression construct. A) Shows a diagram of the construct (not drawn to scale). The SV40 large T antigen was fused in frame at the C-terminus of HSV-1 VP22. Transcription is under the control of the cytomegalovirus promoter (CMV), and the fusion protein contains at its C-terminal end a myc and his epitope tags. The predicted molecular weight of the VP22-large T antigen fusion protein is 120.3 kDa. B) Shows the Western blot analysis of the fusion protein expressed in COS-7 cells. COS-7 cells transfected with VP22-large T antigen plasmid DNA or mock transfected were harvested 48 h after and cell extracts (e) and cell pellets (p) were analyzed by immunoblot with an anti-myc mouse monoclonal antibody (clone 9E10). A band of the expected size of 120 kDa was seen in the transfected cells extract and pellet, albeit more intense in the latter. This might reflect the association of VP22 to microtubular bundles described before (21).

Fig. 3

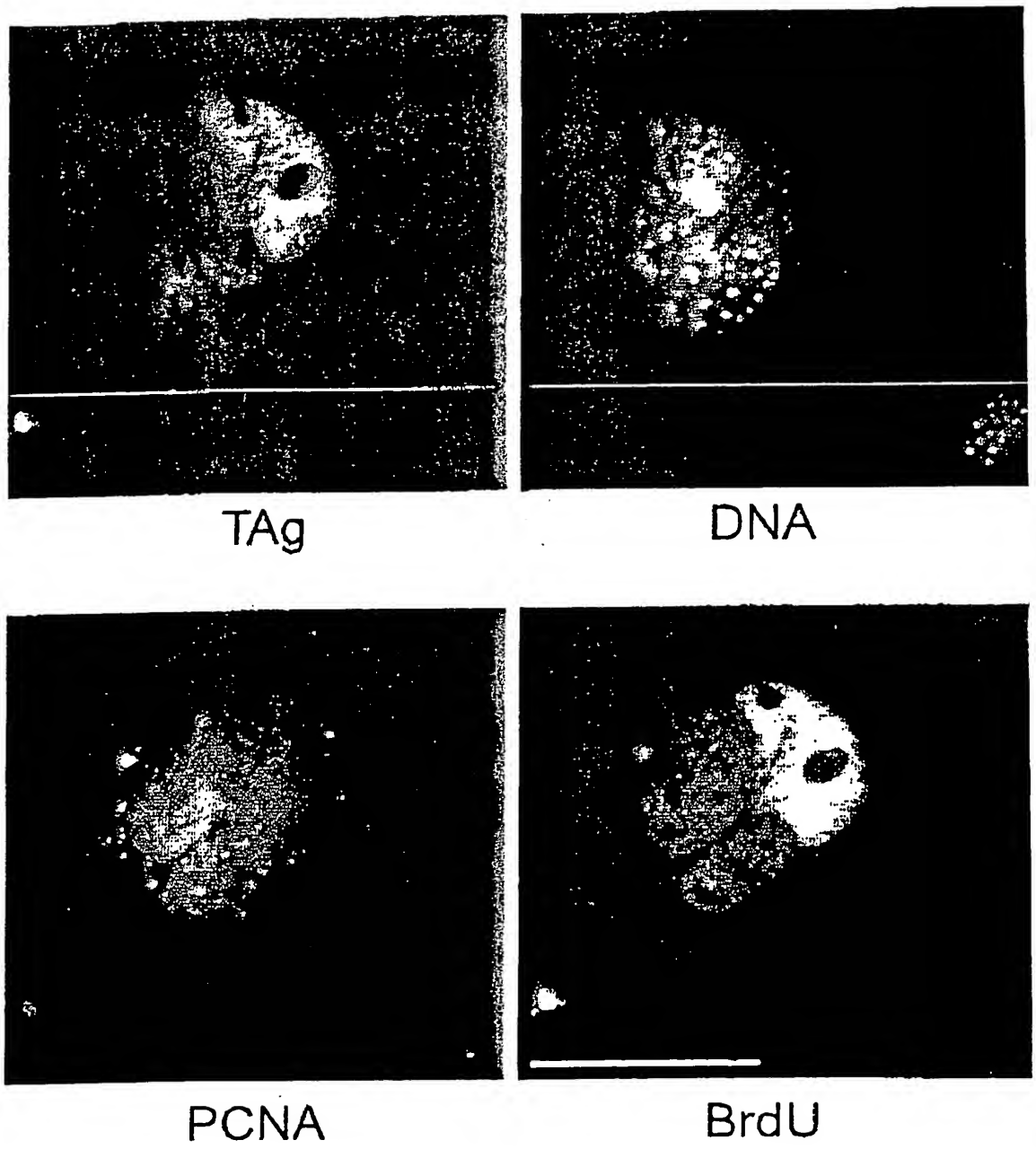

Figure 3. VP22-large T antigen induces S-phase in terminally differentiated muscle cells indicated by induction of proliferating cell nuclear antigen (PCNA) and BrdU incorporation. Mixed cultures, 72 h old, were formaldehyde-fixed and immunostained with antibodies raised against SV40 large T antigen (mouse monoclonal antibody PAB 101), PCNA (rabbit polyclonal antibody, FL 261, Santa Cruz Biotechnology), and BrdU (rat monoclonal antibody, clone BU1/75, Harlan Sera Lab). Total DNA was counterstained with Hoechst 33258. Scale bar 20 µm.

Fig. 4

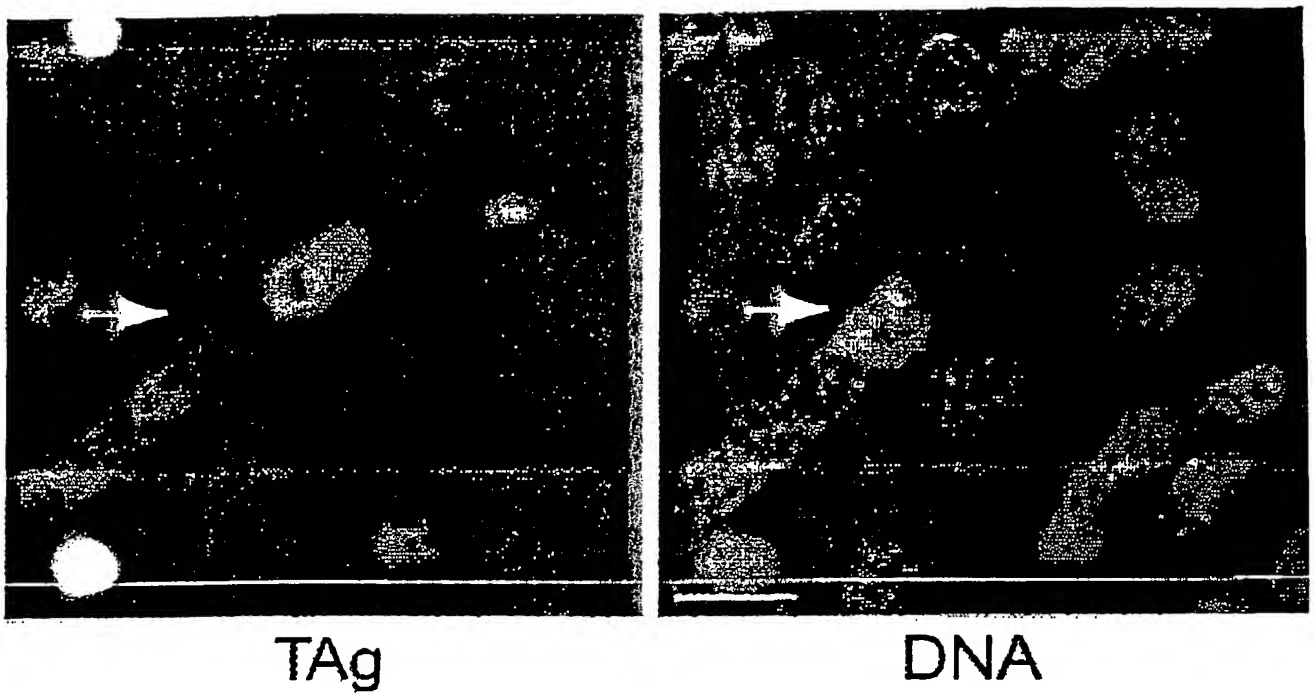

Figure 4. VP22-large T antigen induces mitosis in terminally differentiated muscle cells. Figure depicts a myotube that was cocultured with VP22-large T antigen expressing COS-7 cells for 72 h and stained for SV40 large T antigen and DNA, as described in **Figure 2.** Arrow points to a metaphase plate. Scale bar, 20 μm.